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13. ABSTRACT *(Maximum 200 words)*

The tumor suppressor Rb is thought to be genetically or functionally inactivated in virtually all human cancers including, breast cancer. The Rb protein is negatively regulated by cyclin dependent kinases. The goal of this project is to generate transgenic mice expressing phosphorylation-resistant Rb in the mammary gland and test whether these dominant active alleles of Rb can prevent breast cancer induced by distinct oncogenic pathways in mouse models. In the past year, we have constructed the transgenic plasmids and established 23 MMTV-RbΔK transgenic lines. RT-PCR analysis revealed that some of the transgenic lines expressed various levels of the transgene. We are currently testing the expression of the transgenes in the rest of the lines and study the effect of the transgenes on development of the mammary gland. We will next mate the MMTV-RbΔK transgenic mice with MMTV-myc, MMTV-cyclinD1 and MMTV-neu in order to test whether expression of unphosphorylated Rb can suppress breast cancer in mouse models. If our studies indicate that Rb can prevent or reverse neoplastic growth, future studies will focus on designing strategies to activate the Rb pathway as preventive or therapeutic treatments for breast cancer.

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FOREWORD

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Table of Content:

I. INTRODUCTION

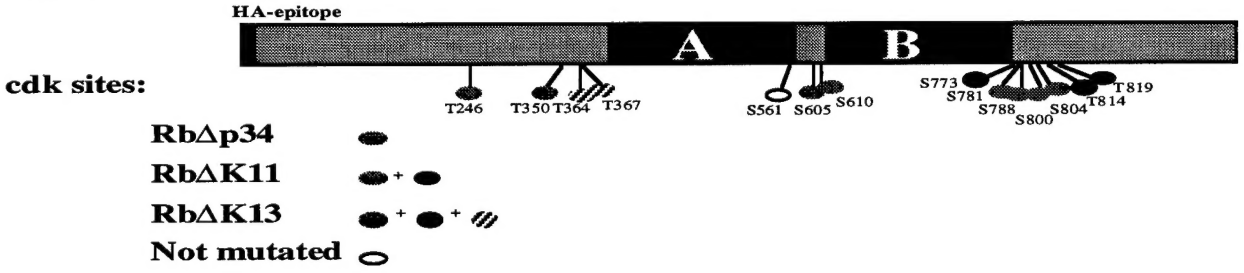
The tumor suppressor Rb controls cell growth, differentiation and survival for modulating the activities of transcription and differentiation factors (9) (8) (7) (11). Rb is either mutated or functionally inactivated in virtually all human cancer including breast cancer. Functional inactivation of Rb occurs by dysregulation of cyclin-dependent kinases (CDKs) that phosphorylate the protein (6). The goal of this project is to assess the effect of blocking functional inactivation of Rb on the development of breast cancer in mouse models (3). To this end we are targeting phosphorylation-resistant (ΔK) alleles of Rb to the mammary gland of transgenic mice using the mouse mammary tumor virus (MMTV) promoter. MMTV-RbΔK transgenic mice will then be mated with other MMTV-onc mice predisposed to breast cancer (1), to test whether unphosphorylated Rb can act as a universal tumor suppressor in the mammary gland.

II. BODY

II.1 *Mutagenesis of phosphorylation sites in Rb*

Fig. 1 shows a schematic structure of Rb, the consensus Ser/Thr CDK-phosphorylation sites and the sites mutated in RbΔK alleles.

Fig. 1: Phosphorylation sites in Rb and RbΔK alleles



These Rb constructs contain a Kozak translation initiation consensus sequence followed by an HA epitope. RbΔp34 has been kindly provided by Dr. P. Hamel, University of Toronto. In this Rb allele, eight phosphorylation sites have been mutated to Ala rendering it hyperactive (2). Nevertheless, transfection of RbΔp34 cannot inhibit Rat1 cell proliferation (4). In contrast, a

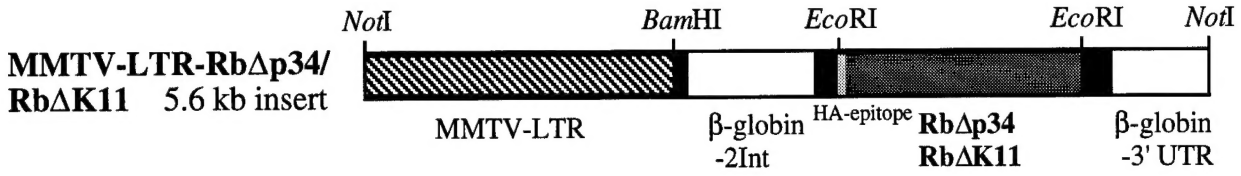
chimeric human-mouse Rb protein in which three additional phosphorylation sites are mutated in the COOH-terminus can inhibit Rat1 cell proliferation (4). We created a similar Rb allele, termed RbΔK11, by mutating S773, T814, T819, in the RbΔp34 backbone. Mutagenesis was accomplished by complex PCR, using Pfu Taq polymerase to reduce the risk of unwanted mutations. Subsequent sequencing of the resulting RbΔK11 clones revealed no fortuitous mutations. To ensure that RbΔK11 is functionally active, we transfected the pECE-HA-RbΔK11 plasmid into COS cells, immunoprecipitated with HA-antibodies and Western blotted with anti-large T antibody, as described (10). We found that RbΔK11 could efficiently bind large T (data not shown). In addition, we transfected pECE-HA-RbΔK11 and pBabe-puro into Saos-2 cells in which transfected Rb induces cell growth arrest and the formation of giant cells (10). We found that pECE-HA-RbΔK11 suppresses growth of Saos-2 cells indicating that the protein is functionally active (data not shown).

We are currently mutating to Ala two additional sites, Thr364 and Thr367, upstream of the A domain of Rb by PCR based mutagenesis to create pECE-RbΔK13 (see **Fig. 1**). This will generate an Rb allele with all CDK sites mutated except for S561 in the pocket domain which we chose not to mutate as it may affect biological activity of Rb.

II.2 ***Construction of MMTV-RbΔK transgenic vectors***

RbΔp34 and RbΔK11 were subcloned into a MMTV expression vector, kindly provided by Dr. R. Coffey (5). The MMTV backbone was subcloned as a XhoI fragment into pBluscript which we modified to contain a unique XhoI site flanked by NotI sites. This allows subsequent excision of the MMTV-RbΔK alleles by NotI digest prior to pronuclear injection. This step was necessary because as opposed to wild-type Rb, RbΔp34 and RbΔK11 contain a XhoI site in their coding sequence. The MMTV-RbΔp34 and -RbΔK11 plasmids are depicted in **Fig. 2**.

Fig. 2. Structure of MMTV-RbΔp34 and -ΔK11 transgenes



To verify that the Rb alleles are correctly expressed and that the MMTV-promoter is hormonally induced, the MMTV-RbΔK plasmids were transfected into COS cells together with a plasmid expressing the glucocorticoid receptor. Transfected cells were treated with dexamathasone (Dex) for 24 hrs. Dex treated cultures expressed the Rb alleles as was revealed by immunostaining and IP/Western blots. Control cultures that did not receive Dex, did not express Rb (data not shown).

II.3 *Generation of MMTV-RbΔK transgenic mice*

The MMTV-RbΔp34 and -RbΔK11 plasmids were digested with NotI to release the transgene from the vector DNA, and purified from gels using a Geneclean extraction kit followed by Elutip column. The purified DNA was micro-injected into fertilized nuclei at the Transgenic Mouse Facilities at the Hospital for Sick Children and The Ontario Cancer Institutes. We routinely check the DNA from the first litter by Southern blot hybridization (e.g. **Fig. 3**); all subsequent genotyping has been done by PCR analysis using primers for Rb (exon 23) and β-globin exon-2 (not shown).

So far, we generated 16 MMTV-RbΔp34 and 7 MMTV-RbΔK11 transgenic founders and obtained successful germ line transmission for all of them. The MMTV-RbΔp34 mice were created and analyzed first. These mice are on a mixed background and are currently crossed into FvB background. The MMTV-RbΔK11 mice are already on a pure FvB background.

II.4 *Expression analysis of MMTV-RbΔK transgenic mice*

To assess the level of expression of the Rb transgenes, MMTV-RbΔp34 females were sacrificed at different developmental stages and total RNA was extracted from mammary glands #4 with the Trizol reagent (Gibco BRL). The RNA was either (a) purified on a poly-dT column

(Qiagen) for Northern blots or (b) re-extracted with Trizol to minimize contaminating DNA and used for RT-PCR. **Fig. 4** shows a Northern blot of RNA isolated from MMTV-RbΔp34 clone #24 virgin female. A 4.6 kb band corresponding to endogenous Rb as well as a weaker 3.6 kb fragment corresponding to the transgene are observed. RNA from a non-transgenic control mouse did not express the 3.6 kb band. Since MMTV transgenes are expressed exclusively in the mammary epithelium whereas Rb is expressed in other breast tissues, including adipocytes (our unpublished in situ hybridization data), the level of the transgene relative to endogenous Rb in the mammary epithelium is higher than appears from the blot.

We subsequently optimized conditions to detect Rb transgene transcripts by RT-PCR. Lines #27 and #30 express the transgene, whereas two other lines do not (**Fig. 5** and data not shown). This simplified RT-PCR approach is currently being used to screen all F1 transgenic lines for expression of the Rb transgene at different developmental stages of the mammary gland. Lines expressing highest levels of the transgenes will be further analyzed for expression by Northern Blots, IP/Western blots and immuno-histochemistry with anti-HA antibodies. The effect of ectopic expression of unphosphorylatable Rb will then be analyzed by whole mount hematoxilin staining and cross sections at different stages of mammary gland development. The ability of the MMTV-RbΔp34 and -RbΔK11 transgenic females to lactate will also be determined. This gross analysis will be accompanied by additional molecular analysis such as BrdU incorporation and PCNA staining to assess proliferation index, and marker analysis depending on the phenotype of the transgenic mice. Highly expressing MMTV-RbΔp34 and -RbΔK11 transgenic mice will be genetically crossed with MMTV-onc mice as outlined in the proposal.

III. CONCLUSIONS

In the past year, we have made the expected progress in our project. We have constructed transgenic plasmids expressing unphosphorylatable Rb and verified that the RbΔK alleles are functionally active. We made constructs in which increasing numbers of Ser/Thr CDK sites in Rb were mutated so we can assess whether partial or complete protection of Rb from phosphorylation is required *in vivo* to block breast cancer. Studies by others revealed the necessity to screen

multiple MMTV transgenic founders to obtain mice with specific, uniform and inducible expression of the transgene. In addition, MMTV transgenic mice were reported to express very low levels of the transgene which is often amplified in tumors (1). We therefore generated a large collection of transgenic MMTV-RbΔK mice and are carefully monitoring their expression levels. The work we accomplished in this first year of grant support and our current analysis will form the basis for the genetic studies. We will mate the MMTV-RbΔK transgenic mice with MMTV-cyclin D1, -myc and -neu, which are often amplified in human cancer and test whether dominant active alleles of Rb can protect from breast cancer initiated by these distinct oncogenes. This analysis will allow us to determine *in vivo* whether inactivation of Rb is an obligatory step in the progression of breast cancer and whether therapeutic approaches directed at activation of the Rb pathway should be attempted for the treatment or prevention of breast cancer.

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V. APPENDICES

Fig. 3 Southern blot analysis of DNA isolated from first litter following micro-injection of MMTV-Δp34. The DNA was digested with SacI, Southern blotted and hybridized with a probe that spans the MMTV vector DNA. The original vector, also digested with SacI, was used as a positive control.

Fig. 4. Northern blot analysis on 2 ug of polyadenylated RNA extracted from whole mammary glands of transgenic MMTV-Δp34 clone #24 and control littermate. Endogenous and transgenic Rb species are indicated. Note that expression of transgenic Rb is from mammary epithelial cells whereas endogenous Rb is expressed in different mammary gland cell types.

Fig. 5. RT-PCR analysis of MMTV-Δp34 . Two and three independent mice from lines #27 and #30 are shown, respectively. RT, reverse transcriptase.

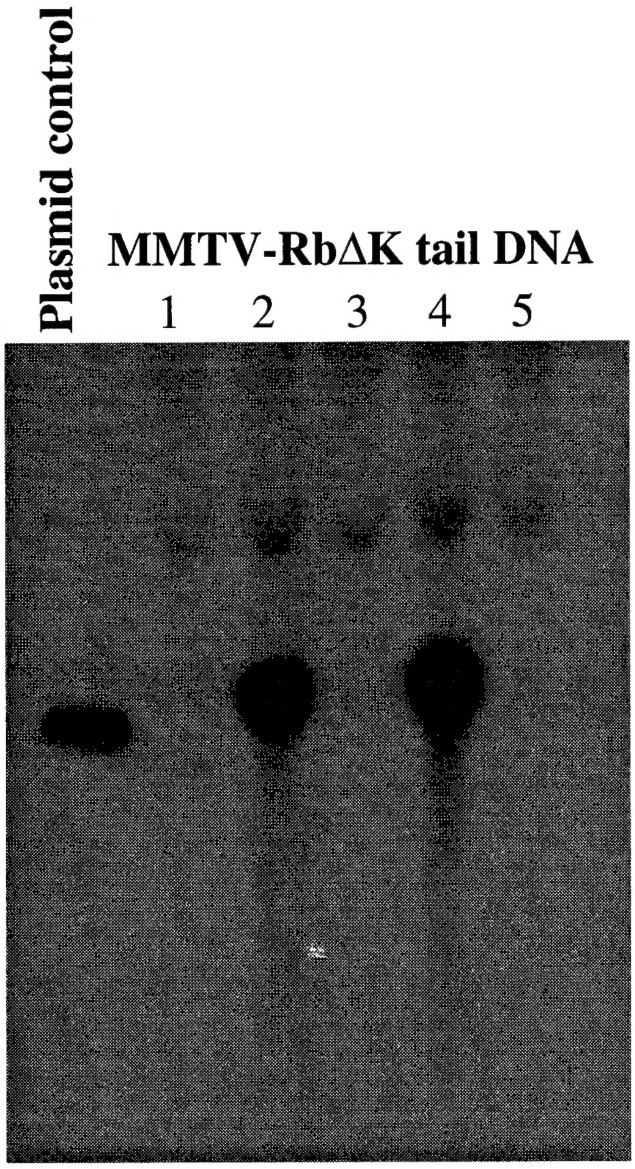

Fig. 3

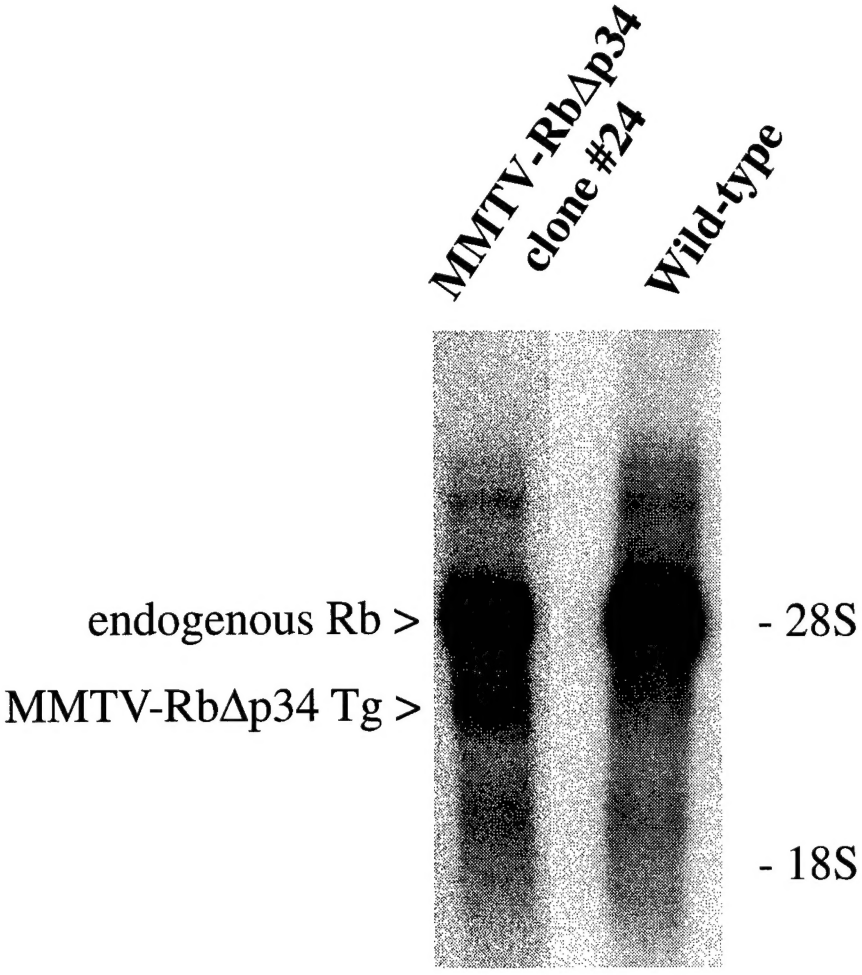
MMTV-RbΔp34
clone #24
Wild-type
endogenous Rb >
MMTV-RbΔp34 Tg >
- 28S
- 18S

Fig. 4

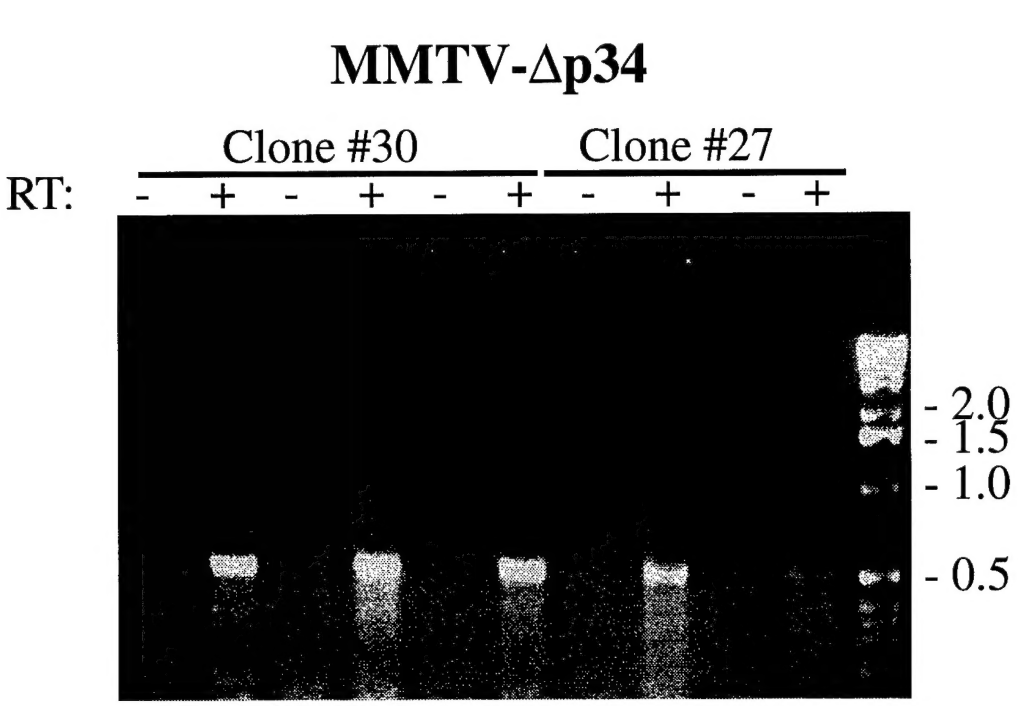

Fig. 5